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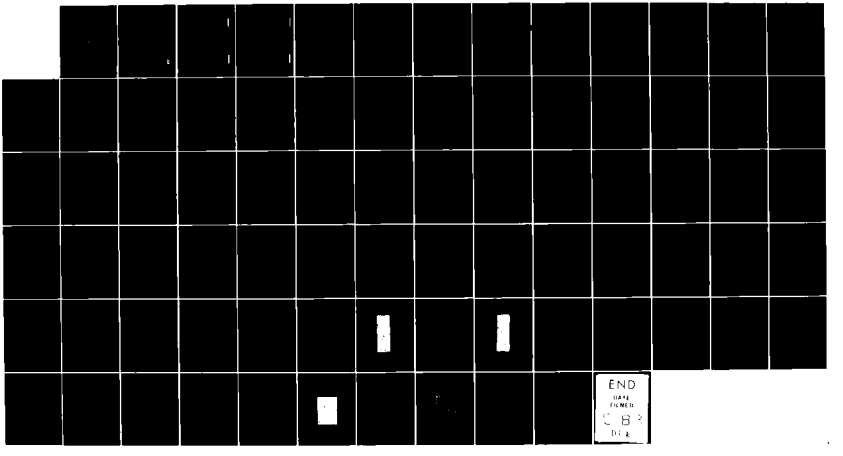

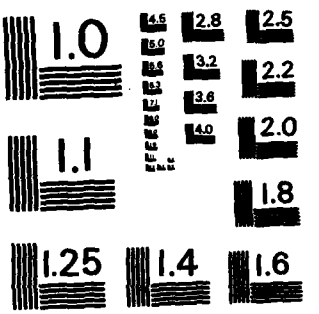

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Report Number:

ROLE OF CELLULAR COMPONENTS OF MOSQUITO CELLS IN VIRAL REPLICATION AND TRANSMISSION

ANNUAL SUMMARY REPORT

Robert H. Schloemer

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Several mosquito cell proteins have been identified as being associated with (or incorporated in) Banzi virus grown in the polyclonal cell line of A. albopictus cells. This fact has been demonstrated by several observations. First, Banzi virions purified by polyethylene glycol precipitation and equilibrium density sucrose gradient centrifugation contain four mosquito cellular proteins. Second, anti-mosquito cell serum aggregates mosquito cell-grown Banzi virus, but not Banzi virus propagated in BHK cells. Third, anti-mosquito cell serum blocks the agglutination of erythrocytes by either unpurified or

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20. (continued)
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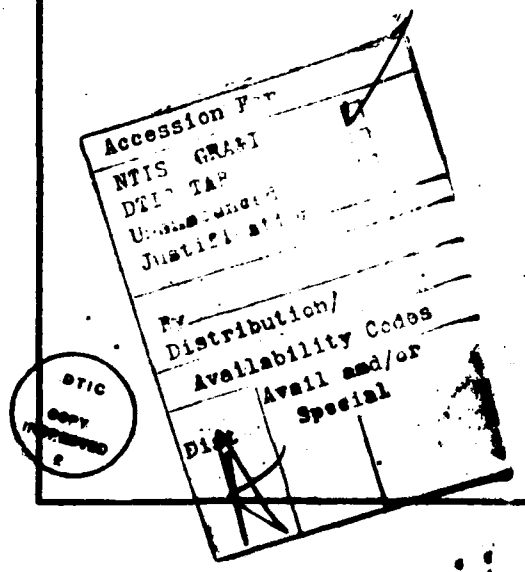
Uninfected mosquito cells secrete a complex of proteins which is able to agglutinate goose erythrocytes. The density of the complex and the pH optimum of its hemagglutinating activity are different than that of Banzi virus grown in mosquito cells for 24 hours. The same proteins (based on molecular weight determinations) which comprise the A. albopictus hemagglutinin are found in purified Banzi virus grown in mosquito cells.

Upon prolonged infection of mosquito cells with Banzi virus, the progeny virions differ in several respects. An apparent increase in the amount of one cellular protein is accompanied by a devrease in the amount of the viral hemagglutinin (V_3). A change in the pH optimum of agglutination of erythrocytes by Banzi virus is observed. An increased reactivity of virus with anti-mosquito cell serum, in terms of neutralization of viral infectivity, is accompanied by a decrease in the ability of anti-Banzi viral serum to inhibit the hemagglutination activity and infectivity of Banzi virus.

As a result of the incorporation of mosquito cell proteins into Banzi virions, mice immunized with uninfected mosquito cells or with purified A. albopictus hemagglutinin are protected against death due to Banzi viral infection. This protection is not due to any component of the medium and is specific for mosquito cell-grown Banzi virus. The protective effect afforded mice by prior immunization with mosquito cells is also observed after challenge with other togaviruses, but not with bunya- or rhabdo-viruses. Furthermore, immunization of mice with a monoclonal cell line of A. albopictus cells also protected mice against death due to Banzi viral infection.

Accompanying the changes in protein profile of Banzi virus and in immunological reactivities of the virus is the appearance of soluble proteins present in medium of mosquito cells infected with Banzi virus. These soluble proteins appear to be of viral origin, prevent Banzi viral production and decrease the number of mosquito cells producing Banzi virus. This antiviral agent is specific for Banzi virus and has no effect on the replication of other togaviruses.



Summary:

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Uninfected mosquito cells secrete a complex of proteins which is able to agglutinate goose erythrocytes. The density of the complex and the pH optimum of its hemagglutinating activity are different than that of Banzi virus grown in mosquito cells for 24 hours. The same proteins (based on molecular weight determinations) which comprise the A. albopictus hemagglutinin are found in purified Banzi virus grown in mosquito cells.

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Accompanying the changes in protein profile of Banzi virus and in immunological reactivities of the virus is the appearance of soluble proteins present in medium of mosquito cells infected with Banzi virus. These soluble proteins appear to be of viral origin, prevent Banzi viral production and decrease the number of mosquito cells producing Banzi virus. This antiviral agent is specific for Banzi virus and has no effect on the replication of other togaviruses.

The temporal relationship between (1) the change in the structure of Banzi virus during prolonged times of replication, (2) the production of a soluble viral protein capable of inhibiting Banzi viral replication and (3) the evolution of acutely infected mosquito cells into a population of persistently-infected mosquito cells suggests that the incorporation of host cell proteins into Banzi virus, and its subsequent effect is related to the establishment of a persistent state of viral infection in mosquito cells.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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LIST OF ILLUSTRATIONS

PROGRESS REPORT

This report covers the period from March 1, 1978 to July 25, 1979.

A. Background and Statement of Problem

In nature, arboviruses are transmitted to vertebrates via arthropod vectors. Replication of flaviviruses in invertebrate hosts is poorly understood and differs remarkedly from growth of flavivirus in mammalian cells. In particular, infection of mosquito cell lines with flaviviruses generally results in a low-titered, non-cytolytic, persistent infection which is in marked contrast to the destructive effects that these viruses have on vertebrate cell cultures. It is of interest that this response closely resembles the in vivo infections of mosquitos in which arboviral infections are generally asymptomatic and life-long.

Replication of flaviviruses in A. albopictus cells differs from its replication in vertebrate cells in the following known ways. Progeny virus released from infected mosquito cells consists of only complete virus (RHA), a fact which is in contrast to the formation of both RHA and SHA in mammalian cells. In addition, progeny virions from infected mosquito cells are altered antigenically, probably as a result of host-cell mediated modification of the viral envelope, in that the hemagglutination (HA) activity of the A. albopictus cell-grown virus is blocked by antisera to uninfected A. albopictus cells. This result suggests that as flaviviruses mature and are released from mosquito cells, a host cell component becomes associated with the virus. A further observation which tends to support this hypothesis is that antisera against A. aegypti mosquitoes neutralized infectivity of Sindbis virus which were propegated in mosquitoes.

The primary aim of this research project is to define the role of host cell components in the transmission and replication of viruses which are capable of growing in both, arthropod and mammalian cells. Specific questions which were posed are as follows.

1) Do arboviruses incorporate host cell components into the virion as they replicate in mosquito cells?

2) If host cell components are incorporated into (or associated with) virions, is there any biological significance as related to transmission and replication of the virus?

3) What is the identity of the host cell components?

4) Is the incorporation of host cell components into virions related to the establishment of viral persistence in mosquito cells?

B. Results

1. Mosquito cell cultures. The mosquito cell cultures used in these experiments appear to be devoid of viral contaminants as determined by the following criteria: (1) supernatants from uninfected cells did not contain an agent which replicates in Vero, BHK or, LLC-MK_2 cells as determined by plaque assays at 31 or 37 C. (2) Cocultivation of mosquito cells with either Vero, BHK or LLC-MK_2 cells did not result in the formation of viruses which can be plaqued on or grown in A. albopictus, BHK, Vero or LLC-MK_2 cells. (3) Injection of either culture medium of mosquito cells, or mosquito cells - intact or disrupted - into suckling mice did not result in death. (4) No syncytium formati in uninfected mosquito cells could be observed. (5) Electron microscope obse tions of uninfected mosquito cells or of concentrated culture fluids fail to reveal the presence of any virus-like structures. (6) Attempts to dete viral antigens in uninfected mosquito cells by indirect immunofluorescence assays were unsuccessful (see Table 1 for list of various antisera).

These assays are performed on a monthly basis and, if any contaminant were detected by any of the above procedures, data obtained since the previous contamination studies are disregarded. By following the procedure outlined above, we feel that the data is valid since we have been unsuccessful in detecting the presence of any contaminating viruses in the mosquito cell lines used in these experiments.

Table 2 depicts the different mosquito cell lines assayed for viral contaminants. Only the polyclonal A. albopictus cell line which was obtained from the American Type Culture Collection and the monoclonal cell line of A. albopictus cells were free of detectable contaminating viruses. In the subsequent experiments, the ATCC mosquito cell line was principally used. In those instances where the monoclonal cell line was employed, specific mention of this fact will be made.

2. Naturally occurring hemagglutinin of uninfected A. albopictus cells. On occasions, uninfected A. albopictus cells released a number of proteins into the medium; some of these proteins were capable of agglutinating goose erythrocytes at a pH optimum of 5.8. The production of this cellular hemagglutinin was variable (HA titers between <1:2 to 1:256), occurred in older monolayers, and was inhibited by actinomycin D. The complex of proteins agglutinating erythrocytes has a sedimentation coefficient of 20 to 25 S, and a density of 1.20 to 1.22 g/cc (see Fig 2C). Results of polyacrylamide gel electrophoresis of the cellular hemagglutinin purified by equilibrium density centrifugation revealed the presence of four proteins (Fig 1). These four proteins appear to be glycoproteins since [^{3}H] glucosamine radioactivity was present in each protein. Attempts to detect radiolabeled precursors to DNA and RNA in the purified A. albopictus hemagglutinin were unsuccessful. Thus, the cellular hemagglutinin appears to be a complex of four glycoproteins.

3. Polypeptide composition of Banzi virus grown in various cell lines. Banzi virus grown in BHK-21 or in A. albopictus cells was radiolabeled with [35S] methionine. After 18-24 hours of infection, the extracellular virions were harvested and cellular debris was removed by centrifugation. Virions were concentrated by polyethylene glycol precipitation and subjected to equilibrium density gradient centrifugation for 40 hours. (This time of centrifugation was necessary to allow the complete separation of the cellular A. albopictus hemagglutinin from Banzi virions, and resulted in considerable loss of viral infectivity). Banzi virus which had been propagated in BHK cells (BV_{BHK}) uniformly banded at a density of 1.17 g/cc (Fig 2A). In contrast, Banzi virions grown in A. albopictus cells ($BV_{A.albo}$) were distributed in a heterogeneous fashion and were present in two main regions of the gradient corresponding to densities of 1.22 g/cc and 1.17 g/cc (Fig 2B). Peaks of [35S] radioactivity were coincident with peaks of infectivity (data not shown). The mosquito cell hemagglutinin isolated from uninfected cells exhibited a density of 1.21 g/cc to 1.22 g/cc (Fig 2C).

Virions present in regions of equilibrum density gradients which corresponded to a density of 1.17 g/cc were pooled, precipitated and subjected to electrophoresis on 8.5% polyacrylamide gels. BV_{BHK} contained three proteins designated V_3, V_2 and V_1 (Fig 3A), whereas $BV_{A.albo}$ contained four additional proteins, termed A_0, A_1, A_2 and A_3 (Fig 3B). It is of interest that the purified mosquito cell hemagglutinin (ρ= 1.22) is composed of four proteins which appeared to have the same mobility in gels as those proteins associated with $BV_{A.albo}$ (Fig 3C).

To determine whether the molecular weight of the V_3 protein differ according to the host cell in which Banzi virus were grown, V_3 protein shown in Figures 3A and 3B were eluted from the gel and electrophoresed on 10% polyacrylamide slab gels. In order to achieve maximal resolution, electrophoresis was contained for 1.5 hours after the bromphenol blue dye had reached the bottom of the gel.

The V_3 protein of $BV_{A.albo}$ exhibited a faster mobility than that of BV_{BHK}. The reason for the altered mobility of V_3 of $BV_{A.albo}$ appears to be due to the lack of sialic acid; neuraminidase treatment of the V_3 of BV_{BHK} resulted in an increased mobility of the protein (data not shown).

The molecular weights of proteins found in purified Banzi virions grown in different cell lines and of proteins of the mosquito cellular hemagglutinin were estimated by comparing their electrophoretic mobilities to those of proteins of known molecular weights. A summary of the molecular weights is given in Table 3.

4. Banzi virus grown in mosquito cells contain cellular proteins. $BV_{A.albo}$ which had been labeled with [35S] methionine was purified by equilibrium density centrifugation. Virions banding at a density of 1.17 g/cc were isolated, disrupted with detergents and incubated with either anti-Banzi viral serum or anti-mosquito cell serum. The resulting immune precipitates were collected, and analyzed by polyacrylamide gel electrophoresis. The results demonstrated that antiviral serum precipitated proteins designated as V_3, V_2, V_1 (Fig 4A). Identical results were obtained when BV_{BHK} were used (data not shown). Antiserum to mosquito cells precipitated proteins referred to as A_0, A_1, A_2 and A_3; no viral proteins were detected in the immune precipitates (Fig 4B). These results suggest Banzi virus which had replicated in A. albopictus cells contained host cell proteins.

5. Ability of anti-mosquito cell serum to aggregate Banzi virus. To demonstrate that mosquito cellular proteins were associated with purified $BV_{A.albo}$, immune aggregation experiments were performed using [^{35}S] methionine labeled virus which had been purified by equilibrium density gradient centrifugation. Anti-Banzi viral serum precipitated intact virus grown in BHK-21 cells or in A. albopictus cells but did not aggregate the A. albopictus hemagglutinin (Table 4). Antiserum against mosquito cells precipitated native mosquito cell hemagglutinin and those intact Banzi virions grown in mosquito cells but did not react with BV_{BHK}. Results of polyacrylamide gel electrophoresis of aggregates which had formed after incubation of $BV_{A.albo}$ with either antiviral serum or anti-mosquito cellular serum demonstrate the same viral and cellular proteins were found in aggregates formed by the addition of antiviral serum (Fig 5A) or of anti-mosquito cell serum (Fig 5B) to intact $BV_{A.albo}$. Polyacrylamide gels of aggregates formed after incubation either of BV_{BHK} with antiviral serum or of anti-mosquito cell serum with the A. albopictus hemagglutinin are similar to Fig 3A and 3C respectively. Thus, these results provide further evidence that mosquito cellular proteins are associated with Banzi virus.

The ability of the A. albopictus hemagglutinin to bind to or stick to Banzi virus was examined by immune aggregation experiments. We reasoned that if the presence of mosquito cellular proteins with purified $BV_{A.albo}$ was due to the binding of the mosquito cell hemagglutinin to extracellular Banzi virus, then exogeneously added A. albopictus hemagglutinin should bind to Banzi virus and should be precipitated by the anti-viral serum. Therefore, labeled mosquito cellular hemagglutinin was incubated with purified, unlabeled $BV_{A.albo}$ or BV_{BHK} prior to the addition of antiviral serum . Alternatively, labeled BV_{BHK} were incubated with unlabeled, purified mosquito cell hemagglutinin prior to treatment with anti-mosquito cellular serum. The mixtures were centrifuged and any precipitates were analyzed for radioactivity. As shown in Table 4, the addition of antiviral serum to those mixtures containing labeled mosquito cell hemagglutinin and unlabeled virus did not result in the precipitation of any radioactivity. These data suggest that exogeneously added mosquito cell hemagglutinin did not associate with Banzi virus grown either in BHK cells or in A. albopictus cells. The fact that labeled BV_{BHK} preincubated with the mosquito cell hemagglutinin was not precipitated by anti-mosquito cellular serum also indicates the inability of the mosquito cell hemagglutinin to bind to the surface of Banzi virus.

6. Adsorption of Banzi virus onto erythrocytes. Another method which had been employed to determine whether the mosquito cell hemagglutinin adsorbed onto surfaces of Banzi virus was selective adsorption onto erythrocytes. Mosquito cell hemagglutinin bound to erythrocytes at pH 5.8, but not at pH 6.5 whereas purified $BV_{A.albo}$ agglutinated erythrocytes at pH 6.5 and not at pH 5.8 (see Fig 6A). Thus, the ability of exogeneously added mosquito cell hemagglutinin to bind to Banzi virus could be ascertained by selective absorption onto goose erythrocytes at either pH 6.5 and pH 5.8. Therefore, [^{35}S] methionine labeled banzi virus was incubated in the presence or absence of mosquito cell hemagglutinin with an excess of goose erythrocytes at the indicated pH. After 1 hour of incubation, erythrocytes were pelleted and the amount of cell-associated radioactivity was determined. Table 5 shows the results of such an experiment.

Banzi virus grown either in BHK-21 cells or in A. albopictus cells attached to erythrocytes only when the pH of the incubation mixture was 6.5; the A. albopictus hemagglutinin adsorbed onto erythrocytes at pH 5.8. When labeled

Banzi virus was mixed with unlabeled mosquito cell hemagglutinin prior to incubation with erythrocytes at pH 5.8, radioactivity could not be detected in the pelleted erythrocytes. Yet, Banzi virus did adsorb onto erythrocytes in the presence of the mosquito cell hemagglutinin when assays were conducted at pH 6.5. In those experiments in which labeled A. albopictus hemagglutinin was incubated with unlabeled Banzi virions, no radioactivity was found to be associated with erythrocytes after incubation at pH 6.5.

These results provide further evidence that Banzi virus do not attach to the mosquito cell hemagglutinin. These data may suggest that the host cell proteins present in Banzi virus grown in A. albopictus cells are structural components of the virion.

7. Variation in polypeptide composition of Banzi virus grown in A. albopictus cells. Banzi virus was labeled with [^{35}S] methionine from 0-24 hours, 6-7 days or 59-60 days after infection of mosquito cells. The medium which contained extracellular virus was clarified by low-speed centrifugation, and then subjected to centrifugation at 125,000 x g for 4 hours. The pellets which contained the virions were resuspended in PBS and incubated with anti-Banzi viral serum. The resulting immune aggregates were collected and analyzed by polyacrylamide gel electrophoresis. The relative amounts of individual proteins in each viral preparation were normalized to the amount of V_2 present and are shown in Table 6.

The relative amounts of all proteins except for V_3 and A_1 were essentially the same in Banzi virus grown for 0-1 day, 6-7 day and 59-60 days in A. albopictus cells. A decrease in the amount of V_3 relative to V_2 was observed in virus which had been labeled after 6 days of infection. After 60 days of infection, V_3 could not be detected in Banzi virions. Concommitantly, an increase in the relative amounts of A_1 to V_2 was detected. The fact that the ratio of other cellular proteins (A_0, A_2 and A_3) to V_2 remained constant suggests a specific increase in the amount of only one cellular protein (A_1) in Banzi virus. Whether the increase in the relative amounts of A_1 protein reflects an increase in the A_1 protein and/or a proteolytic cleavage product of V_3 which has the same molecular weight as the cellular protein (A_1) is unknown.

8. Change in pH optimum of Banzi viral hemagglutination. The change in the relative amounts of V_3 in Banzi virus is accompanied with a shift in the pH optimum of Banzi viral hemagglutination. Purified Banzi virus grown in mosquito cells for 1 day has a pH optimum for hemagglutination at 6.5 (Fig 6A). In contrast, virus grown in mosquito cells for 7 days exhibited HA activity at pH 5.8 and 6.5; the highest titer was obtained at pH 5.8 (Fig 6B). The presence of HA activity at pH 5.8 is not due to free A. albopictus HA since the virus used in these experiments were isolated from equilibrium density at a density of 1.17 g/cc (see Fig 3). These results may indicate that the A. albopictus hemagglutinin substitutes as the functional hemagglutinin on the virus and may replace the V_3 protein in the assembly of the virus. Virus which had been grown for 60 days in mosquito cells could not be obtained in sufficient quantities to perform these assays. However, purified Banzi virus grown in mosquito cells for 12 days was obtained in sufficient amounts to detect HA activity. As shown in Fig 6C, HA activity was detected only at pH 5.8. In a preliminary experiment, polyacrylamide gel electrophoresis of [^{35}S] methionine labeled virus grown for 12 days which had been purified by equilibrium density gradient showed the absence of V_3 (data not shown).

9. Effects of anti mosquito cell serum on hemagglutination and infectivity of banzi virus. Antiserum to uninfected A. albopictus cells were prepared in rabbits in the following manner. A. albopictus cells (2×10^8) were washed six times with warm PBS and disrupted by dounce homogenization. The nuclei were removed by low-speed centrifugation and the supernatant mixed with Freund's complete adjuvant was injected into the ear vein. After six weekly injections, serum was collected and complement was destroyed by heat treatment (56C for 30 minutes). The titer of this serum as determined by double diffusion in Outerlony plates was 1:64. The serum also contained antibodies to fetal calf serum; these antibodies were removed by adsorption to fetal calf serum. Experiments were conducted to determine whether this antiserum could inhibit viral hemagglutinating (HA) activity and viral infectivity. These experiments include unpurified and purified virus derived from various cell lines.

Tables 7 and 8 show the effect of various antisera on the hemagglutinating ability of virus grown in BHK or in A. albopictus cells. The fact that antiserum to A. albopictus cells inhibited the HA activity of Banzi virus derived from mosquito cells indicates that host cell proteins are associated with Banzi virus grown in mosquito cells (Table 7). Anti-mosquito cell serum had no effect on the HA activity either of virus grown in BHK cells or in mosquito cells and then passaged twice in BHK cells. Of interest is the fact that anti-Banzi viral serum.did not inhibit the HA activity of virus grown in mosquito cells but did block the HA activity of virus grown in BHK cells (Table 7). These results confirms the hypothesis that the surface of Banzi virus grown in mosquito eells is modified by the presence of mosquito cellular proteins.

Similar results were obtained using purified Banzi virus (Table 8). In this case, the ability of various antisera to inhibit the hemagglutinating activity of Banzi virus grown for 7 days in A. albopictus cells could to examine at the two pH optima of hemagglutination (pH 5.8 and pH 6.5). The ability of anti-mosquito cell serum to inhibit the viral hemagglutinin (pH 6.5) could be explained simply by a steric inhibition phenomena. However, the reason for the inability of antiviral serum to inhibit the HA activity observed at pH 6.5 is unclear.

To demonstrate that the inhibition of HA activity of mosquito cell grown virus by anti mosquito cell serum was due to the interaction of antibodies with host cell proteins on the virus, anti-mosquito cell serum was treated in a variety of ways prior to determining its ability to inhibit viral hemagglutination. The virus used in these experiments were purified Banzi virus grown in mosquito cells for 24 hours (A.al 1). Table 9 shows that the ability of the serum to inhibit viral HA activity is abolished upon pretreatment with either intact mosquito cells or with purified A. albopictus hemagglutinin, results which indicates that the inhibition of viral HA activity is due to the interaction of antibodies with cellular proteins on the virus. Furthermore, the fact that pretreatment of sera with trypsinized mosquito cell hemagglutinin did not abolished the ability of the sera to inhibit viral HA activity indicates that these antibodies react with a cellular protein (and not with glycolipids) on the virus.

The ability of anti-mosquito cell serum to neutralize the infectivity of Banzi virus grown in mosquito cells was investigated by two methods: 1) survival of mice injected with Banzi virus mixed with anti-mosquito cell serum and 2) plaque neutralization assays. Table 10 shows the number of mice which died within 21 days after virus challenge. Virus grown in A. albopictus cells for 1, 7 or 60 days were incubated with antiserum or PBS for 30 minutes at room temperature before injection into mice. As the results indicate, prior incubation of Banzi virus grown in mosquito cells for 7 days or 60 days with antiserum resulted in protection of mice against a 10 LD_{50} dose of virus. Results of experiments utilizing virus propagated in cell for 1 day were variable. Table 11 shows similar results when virus which had been purified by buoyant density centrifugation were employed.

Table 12 shows the effect of anti-mosquito cell serum on the infectivity of Banzi virus grown in various cell lines. In these assays, the end point was calculated as the highest dilution of sera which caused the neutralization of 50% of plaques. All sera were heat-inactivated (56C for 30 minutes) to destroy complement. The significant findings are 1) antiserum to mosquito cells neutralized the infectivity of only virus propagated in A. albopictus cells; it had no effect BV_{BHK} or on virus grown in mosquito and then passaged in BHK cells; 2) Anti-BHK cellular serum did not neutralize the infectivity of virus grown in any cell line and 3) a difference in the ability of anti-mosquito cell serum to neutralize the infectivity of virus grown for 1 day or 7 days in mosquito cells is observed (The difference in the neutralization titer of anti-mosquito cellular serum against virus grown 7 days or 60 days is not significant). Table 13 shows similar results of identical experiments utilizing purified virions.

Table 14 demonstrates that antibodies present in antimosquito cell serum which neutralized viral infectivity are directed against mosquito cellular proteins. Precubation of the antiserum with mosquito cells or with purified mosquito cell hemagglutinin resulted in the loss of the ability of the anti-serum to neutralize viral infectivity. Furthermore, the neutralizing antibodies are directed against the cellular proteins on the virus since incubation of anti-mosquito cell serum with trypsinized mosquito cell proteins had no effect on the ability of the antiserum to neutralize viral infectivity.

10. Mouse protection experiments. Since the possibility of employing A. albopictus cells as a vaccine against infection by Banzi virus was being examined in a mouse system, the induction of antibody response to A. alhopictus cells was examined. Mice were injected intraperitoneally or intravenously with 10^7 washed, disrupted A. albopictus cells once a week for 6 weeks. Sera were collected from mice 7 days after the last injection. The assay to detect the presence of antibodies to A. albopictus cells was to determine the ability of serum to inhibit the hemagglutinating activity of Banzi virus grown in A. albopictus cells. The results which are shown in Table 15 indicate that the route of administration of the immunogen did not influence the amount of antibodies produced and that five weekly injections were required to result in maximal antibody response. In a preliminary experiment, the mouse anti-mosquito cell serum neutralized the infectivity of mosquito cell grown virus (Titer 1:1024).

The ability of antibodies against A. albopictus cells to protect mice against infection by Banzi virus grown for seven days in A. albopictus cells was determined. Mice were immunized i.p. once a week for 5 weeks with either PBS (control) or A. albopictus cells which had been washed with PBS prior to disruption. One week later, mice were challenged i.p. with 1, 10 or 100 LD_{50} of Banzi virus. The results illustrated in Table 16 show the number of mice which survived 21 days after challenge.

These results demonstrate that immunization of mice with uninfected mosquito cells does protect against infection with mosquito cell grown virus.

Table 17 is a summary table illustrating the ability of various immunogens to protect mice from virus challenge employing 100 LD_{50} of virus grown seven days in A. albopictus cells or 24 hours in BHK cells. As noted previously, immunization of mice with A. albopictus cells results in protection against challenge by A. albopictus cell-grown virus, and not by BHK cell-grown virus. Prior immunization of mice with BHK cells does not afford protection against virus challenge. The observed protection against challenge with mosquito cell grown virus in mice which were previously immunized with mosquito cells is not a result of a component of the medium adhering to both virus and cell surfaces because of the lack of protection to virus challenge in mice immunized with media used to cultivate A. albopictus cells. Of significent interest is the observation that mice immunized with purified A. albopictus hemagglutinin are protected against death caused by mosquito cell grown virus.

To determine whether immunization of mice with A. albopictus cells will protect mice against death by other viruses capable of replicating in mosquito cells, experiments similar to those described above were conducted employing Eastern equine encephalitis virus (alphavirus), Japanese encephalitis virus (flavivirus), Germiston virus (bunyavirus) and vesicular stomatitis virus (rhabdovirus). The results illustrated in Table 18. demonstrate that mice immunized with mosquito cells survived challenge with Eastern equine encephalitis and Japanese encephalitis viruses. All other permutations of (i) cell line used as the immunogen (ii) challenge virus and (iii) cell line employed for viral propagation resulted in death of mice.

To ascertain whether the observed protection against togaviral challenge is "unique" to the ATCC A. albopictus cell lines, similar protection experiments were performed with a monoclonal A. albopictus cell line. Preliminary experiments indicate that this monoclonal cell line produces a cellular hemagglutinin whose pH optimum is 5.8. As shown in Table 19, immunization of mice with the monoclonal cell line also resulted in protection of mice against death due to Banzi virus.

11. Characterization of mosquito cells infected with Banzi virus. Since the polypeptide composition of Banzi virus changed as virus replicate for longer time periods in mosquito cells, we investigated the possibility that this change is related in some manner to the development of viral persistence. Therefore, the growth of Banzi virus in mosquito cells was examined for a period of 91 days after infection. The total amount of virus released from cells was determined at regular intervals. In addition, the number of cells secreting virus was ascertained by infectious center assays. Figure 7A shows that the titer of virus declines as the length of time of infection increases. Similarly, a decrease in the number of cells secreting infectious virus occurs . After 21 days of infection, the titer of virus and number of infectious centers re-

mained relatively constant (Fig 7B). In other experiments not reported here, cells infected for 60 days appear to have the same gross morphology and generation time as uninfected mosquito cells.

To ascertain whether mosquito cells infected for 60 days were persistently infected with Banzi virus, the ability of these cells to support the replication of superinfecting togaviruses was examined (Table 20). Mosquito cells infected with Banzi virus for 60 days excluded the replication of superinfecting Banzi virus, but were as susceptible to infection by Eastern equine encephalitis virus or Japanese encephalitis virus as uninfected (fresh) mosquito cells. Since cells infected with Banzi virus for 60 days were resistant to superinfection by the homologous virus, produced less virus than acutely infected mosquito cells and less than 1% of these cells secreted virus, these cells were indeed persistently infected with virus.

Another assay employed to determine whether cells infected for 60 days were persistently infected with Banzi virus was to determine the number of cells containing viral antigens as assayed by indirect immunofluorescence procedures. It was expected that approximately 1% of these cells should contain viral antigens since only 1% of cells produced virus. However, the same number of cells infected with Banzi virus for 60 days contain viral antigens as did acutely infected cells (24 hour postinfection) (Table 20). Uninfected mosquito cells did not react with antiviral serum. The fact that prior adsorption of antiviral serum with infected mosquito cells abolished subsequent fluorescence in acutely infected cells or in Banzi virus-persistently infected (BVPI) cells suggest that this assay is specific for Banzi viral antigens. Figure 8 shows the pattern of fluorescence in acutely infected cells (Fig 8A) and in persistently infected cells (Figure 8B).

12. Soluble viral antigens in medium of mosquito cells persistently infected with Banzi virus. To account for the observation that less than 1% of BVPI mosquito cells secrete virus and approximately 90% contain viral antigens, we proposed that the 1% of cells producing virus also secrete a soluble viral protein into the medium. The viral protein would be alterable to bind to uninfected cells. To test this hypothesis, culture fluids of persistently infected mosquito cells (60 days of infection) and of uninfected mosquito cells were subjected to filtration through an Amicon XM100 filter. The resulting filtrates which contained material of 100,000 daltons or less were incubated with uninfected mosquito cells for 2 hours. The binding of viral proteins in the filtrates to cells was detected by immunofluorescent procedure using antiviral serum. Cells treated with filtrates of medium of uninfected cells did not react with antiviral serum. However cells treated with filtrates of BVPI mosquito cells did react with antiviral serum. Figure 9 A shows that this pattern of fluorescence is indistinguishable from that of persistently infected cells. (Fig 9 B) These results suggest that a viral protein(s) is being secreted by BVPI cells and binds to uninfected mosquito cells.

13. Ability of soluble viral proteins to inhibit Banzi viral replication. Filtrates of mosquito cell growth medium and of medium from uninfected, acutely infected or persistently infected mosquito cells were applied to uninfected cells for 4 hours prior to infection with Banzi virus. The amount of virus present in the medium 24 hours postinfection was determined by plaque assays. Table 22 shows that filtrate of medium of acutely infected cells inhibited virus production by 29% whereas soluble proteins in medium of BVPI mosquito cells decreased virus production by 99.98%. Pretreatment of medium of BVPI mosquito cells with anti-Banzi viral serum but not with anti-mosquito cell serum alleviated the observed inhibition of Banzi viral replication. These results indicate that the soluble viral protein has the ability to inhibit Banzi viral replication.

Figure 10 shows that filtrates of medium of BVPI mosquito cells have the ability to inhibit the attachment of Banzi virus to mosquito cells. However, only a 50% decrease in the amount of virus attaching to mosquito cells occurred. This result suggest that the soluble viral proteins act to inhibit viral production at some step other than attachment of virus to cells.

14. Evidence for two antiviral agents in medium of BV persistently-infected mosquito cells. To determine the size of the viral protein which has the ability to inhibit viral replication, filtrates of medium of BVPI mosquito cells were placed in a dialysis sack which is permeable to molecules of 12,000 daltons or less. The filtrate was then dialyzed against 2 volumes of M and M medium for 18 hours. After this time, the dialysate (material outside the sack) was collected. The dialysis sack was then extensively dialysed against M and M medium. The resulting dialyzed filtrate (contents with the sack) was collected. The original filtrate, dialysate and dialyzed filtrate were assayed for their ability to inhibit Banzi viral replication.

Results shown in Table 23 demonstrates that the original filtrate of medium of BVPI mosquito cells caused a 4 1/2 log decrease in viral replication. The dialysate (material of 12,000 daltons or less) caused a similar degree of inhibition of viral replication. The dialyzed filtrate caused approximately a 50% inhibition of the growth of Banzi virus. Table 24 shows that treatment of medium of BVPI cells with antiviral serum but not with anti mosquito cell serum prior to the preparation of the dialysate alleviated the antiviral activity.

These data indicate that (1) BVPI mosquito cells secrete viral antigens which have the ability to inhibit Banzi viral replication, (2) There appears to be two classes of viral antigens which have a antiviral activity, (3) The viral antigen whose molecular weight is 12,000 daltons or less is more potent in preventing growth of Banzi virus.

15. Properties of the antiviral agent. The following studies were performed with the antiviral agent whose molecular weight is less than 12,000 daltons.

Figure 11 shows the effect of dilution of the dialysate of medium of BVPI mosquito cells on its ability to inhibit growth of Banzi virus. As can be seen, the antiviral activity is lost in a linear and progressive manner with dilution.

Pretreatment of uninfected mosquito cells with dialysates of medium of BVPI mosquito cells results in a decrease in the number of cells producing Banzi virus after 24 hours of infection (Table 25). This antiviral agent inhibits the replication of only Banzi virus; pretreatment of mosquito cells with dialysates of medium of BVPI mosquito cells did not affect the replication of Japanese encephalitis or Eastern equine encephalitis viruses (Table 26). Uninfected mosquito cells pretreated with dialysates of medium of BVPI cells reacted with anti banzi virus serum as determined by indirect immunofluorescence (Figure 12). It is noteworthy to observe that the pattern of fluoresence in these cells is similar to that of persistently infected cells. (See Figure 8B).

These results suggest that the characteristics of persistently infected mosquito cells can be produced by the viral protein present in the dialysate of medium of BVPI cells. Specifically, (1) BVPI cells produce less virus than acutely infected cells; the antiviral agent inhibits Banzi virus production, (2) only 1% of BVPI cells secrete infectious virus; the antiviral agent decreases the number of cells producing virus, (3) approximately 90% of BVPI cells contain viral antigens as determined by immunofluorescence assays; uninfected mosquito cells treated with the antiviral agent reacts with anti Banzi viral serum, (4) BVPI cells are resistant to superinfection by Banzi virus but not by other togaviruses; the antiviral agent inhibits the replication of only Banzi virus.

If the antiviral agent, described above, is responsible for the induction, or maintenance of the persistent state of viral infection, then a progressive appearance of this agent should occur after infection of mosquito cells with Banzi virus. Figure 13 shows the time course of appearance of the anti-Banzi viral activity after infection of mosquito cells. As is evident, the level of anti-Banzi viral activity increased progressively over a 6 day period. However, in the two experiments reported, there appears to be a variation in the amount of anti-Banzi viral activity present at any one time.

C. Conclusions:

1) Banzi virus grown in mosquito cells contain host cell proteins. These host cell proteins are glycoproteins are appeared to be identical to those proteins comprising the mosquito cell hemagglutinin.

2) A change in the polypeptide profile of Banzi virus occurs upon prolonged incubation in mosquito cells. An apparent increase in one cellular protein (A_1) is accompanied by a decrease in a viral protein (V_3). Accompanying this change in proteins are (a) a shift in the pH optimum of hemagglutination by Banzi virus (pH 6.5 → pH 5.8), (b) increased reactivity of Banzi virus with anti-mosquito cell serum, and (c) decrease in the reactivity of Banzi virus with antiviral serum.

3) Accompanying the changes in the protein composition of the virus and in immunological reactivities is the progressive appearance of an antiviral agent in the medium of Banzi virus-infected cells.

4) This antiviral agent appears to be of viral origin, inhibits Banzi viral replication, and decreases the number of cells producing the virus. Furthermore, the antiviral agent inhibits the replication of only Banzi virus; no inhibitory effect on the replication of other togaviruses could be detected.

5) The temporal relationship between the changes in the structure of Banzi virus, in the production of a soluble viral protein able to inhibit viral replication, and in the evolution of acutely infected mosquito cells into a population of persistently infected mosquito cells suggests that the incorporation of host cell proteins into Banzi virus may be related to the establishment of a persistent state of infection.

6) As a result of the incorporation of mosquito cell proteins into Banzi virions, mice immunized with uninfected mosquito cells are protected against infection (death) by Banzi virus propagated in mosquito cells. This protection is not due to any component of the medium and is due, in part, to the humoral response of the mouse to mosquito cell proteins. Since immunization of mice with purified A. albopictus cell hemagglutinin also protects mice from infection by mosquito cell-grown virus, it is likely that the mosquito cell antigen which induces protective antibodies is one or more of the proteins constituting the A. albopictus hemagglutinin. The protective effect afforded mice by prior immunization with mosquito cells is also observed after challenged with other togaviruses, but not with bunya- or rhabdo-viruses. Furthermore, immunization of mice with a monoclonal cell line of A. albopictus cells also afforded protection against viral infection.

D. Research Plans August 1, 1979 to December 31, 1979.

1) Do other arboviruses grown in the polyclonal A. albopictus cell line contain the same host cell proteins as Banzi virus?

2) Does Banzi virus grown in the monoclonal A. albopictus cell line contain the same host cell proteins as Banzi virus propagated in the polyclonal cell A. albopictus cell line? Are the hemagglutinins secreted by the two A. albopictus cell lines identical?

3) What is the identity of the soluble viral protein(s) secreted by mosquito cells persistently infected with Banzi virus? Does this antiviral agent inhibit the replication of Banzi virus in mammalian cells?

Table 1 Antiviral sera used in immunofluorescence assays.

Banzi virus

Eastern equine encephalitis virus

Japanese encephalitis virus

Dengue II virus

La Crosse virus

Germiston virus

Wesselbraum virus

Vesicular stomatitis virus

Chikungunya virus

Table 2 Cell lines assayed for viral contamination

Criteria	Aedes albopictus				Aedes aegypti	
	Singh (ATCC)	Bhat[a] (RML)	Singh[b] (WRAIR)	Monoclonal (D.L. Bishop)	Peleg[c]	Singh[c]
EM	-	+	+	-	+	+
Mice mortality	-	+	+	-	ND	+
PFU, I.C. assays	-	ND	ND	-	+	ND
Immunofluorescence	-	ND	ND	-	ND	ND

a Obtained from Dr. C. E. Yunker, Rocky Mountain Laboratory

b Obtained from Dr. W. Brandt, Walter Reed Army Institute of Research

c Obtained from Dr. J. Pashke, Purdue University

ND Not determined

Figure 1 Electropherogram of proteins associated with the mosquito cell hemagglutinin. Monolayers of uninfected mosquito cells were labeled with [^{35}S] methionine (10 µCi/ml) in M and M medium containing 1/100 the normal amount of methionine. After 24 hours of incubation, the culture fluid was harvested and clarified by centrifugation at 800 x g for 15 minutes. Polyethylene glycol 6000 and NaCl was then added to a final concentration of 8% and 2.3 g/100 ml respectively. After stirring for 1 hour at 4C, the mixture was centrifuged at 10,000 x g for 1 hour. The pellet was resuspended and layered onto a 28 to 50% sucrose gradient. After centrifugation for 40 hours at 76,000 x g, the A. albopictus hemagglutinin present at a density of 1.20-1.23 g/cc was collected, precipitated and subjected to electrophoresis on 8.5% SDS-polyacrylamide gels.

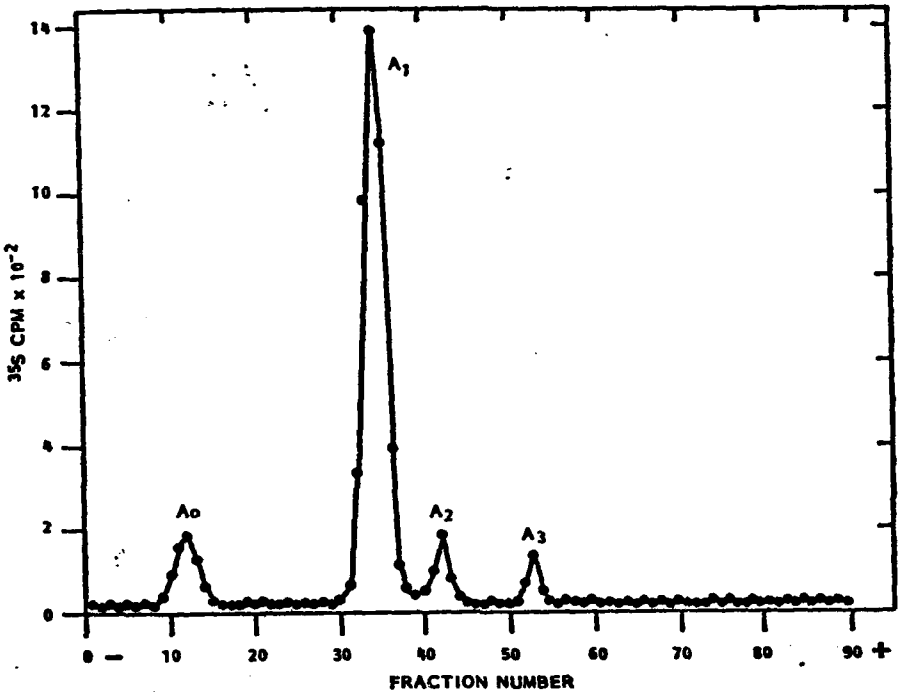
14
12
10
8
6
4
2
0
^{35}S CPM x 10^{-2}
A$_0$
A$_1$
A$_2$
A$_3$
0 — 10 20 30 40 50 60 70 80 90 +
FRACTION NUMBER

Fig. 1

Figure 2 Distribution of Banzi virus grown in BHK-21 cells or in A. albopictus cells in equilibrium density gradients. Banzi virus was grown in BHK-21 cells and A. albopictus cells in the presence of [^{35}S] methionine and 100 mM excess NaCl. An equivalent number of A. albopictus cells was mock-infected and processed in an identical manner. After incubation, the culture medium was clarified by centrifugation at 1500 x g for 15 minutes, and virus was precipitated by polyethylene glycol. The resulting precipitate was resuspended and layered onto a 28 to 50% sucrose gradient. After centrifugation for 40 hours at 76,000 x g, gradients were fractionated into 1-ml aliquots and the presence of virus was determined by plaque assays on monolayers of BHK-21 cells. Location of the mosquito cell hemagglutinin was determined by HA assays at pH 5.8. Approximately 10^{13} PFU of virus were placed on the gradients. (A), Banzi virus grown in BHK-21 cells; (B), Banzi virus grown in A. albopictus cells; (C), medium from uninfected A. albopictus cells.

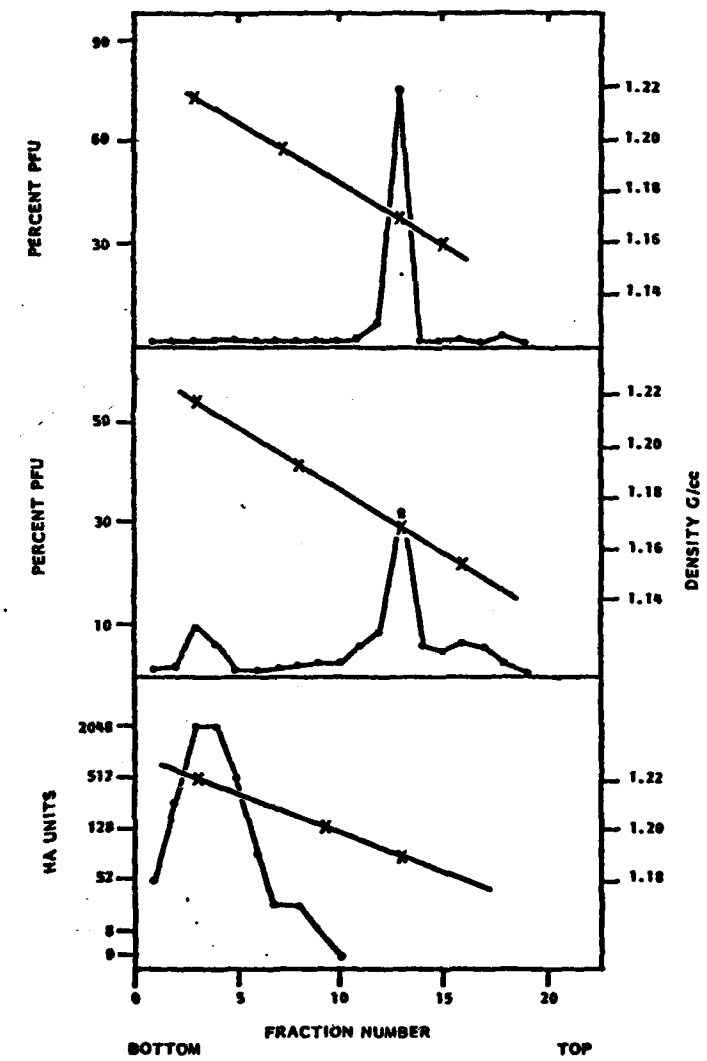
PERCENT PFU
90
60
30
1.22
1.20
1.18
1.16
1.14
PERCENT PFU
50
30
10
1.22
1.20
1.18
1.16
1.14
DENSITY G/cc
HA UNITS
2048
512
128
32
8
0
1.22
1.20
1.18
0
5
10
15
20
FRACTION NUMBER
BOTTOM
TOP

Fig. 2

Figure 3 Electropherograms of polypeptides of Banzi virus. Banzi virus grown in the presence of [^{35}S] methionine was purified by equilibrium gradient centrifugation as described in the legend to figure 2. Regions of gradients corresponding to a density of 1.17 g/cc were pooled and virus was precipitated by trichloroacetic acid. The mosquito cell hemagglutinin which banded at densities of 1.21 to 1.22 g/cc (see Fig 2C) was pooled and treated in a similar manner. (A), peptides of Banzi virus grown in BHK-21 cells; (B),peptides of Banzi virus propagated in A. albopictus cells; (C), peptides of mosquito cell hemagglutinin obtained from uninfected A. albopictus cells.

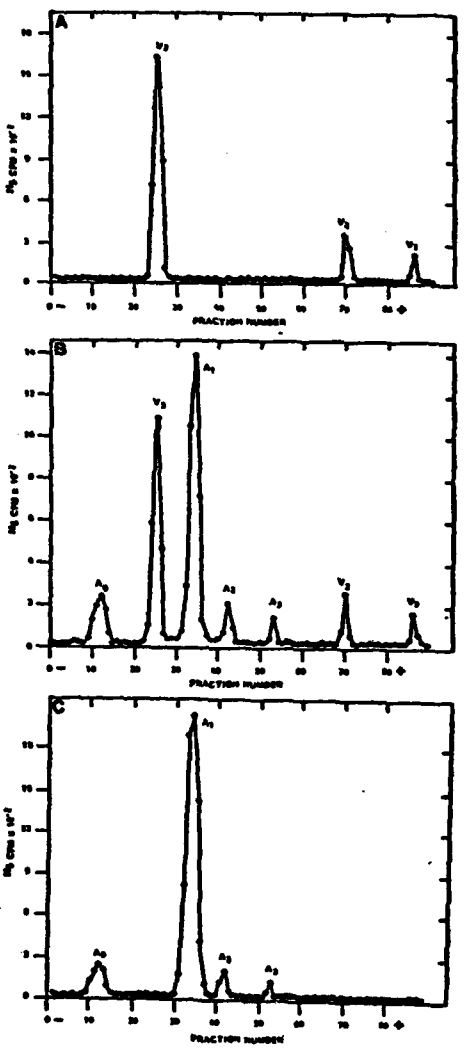

Fig. 3

Table 3 Summary of molecular weights of proteins found in purified Banzi virus grown in various cell lines[a]

Polypeptide	BV_{BHK} (daltons)	$BV_{A.albo}$ (daltons)	A. albopictus hemagglutinin (daltons)
V_3	56,000	53,000	ND
V_2	14,500	14,500	ND
V_1	8,400	8,400	ND
A_0	ND	88,000	88,000
A_1	ND	44,000	44,000
A_2	ND	35,000	35,000
A_3	ND	23,000	23,000

a Molecular weights of peptides were determined by comparison of their electrophoretic mobilities to those of bovine serum albumin, chicken ovalbumin, Trypsin and cytochrome C on 8.5% and 10% SDS-polyacrylamide gels. ND, not detected.

Figure 4 Electrophoretic profiles of polypeptides of Banzi virus precipitated by anti-Banzi viral serum or by antiserum to A. albopictus cells. Purified [^{35}S] methionine labeled Banzi virus grown in A. albopictus was dissociated with NP-^{40} and deoxycholate before addition of antiserum. Immunoprecipitation and electrophoresis was performed as described. Position of proteins was determined by electrophoresis in parallel gels of labeled Banzi virus. (A), polypeptides precipitated by antiviral serum; (B), polypeptides precipitated by antiserum to uninfected mosquito cells.

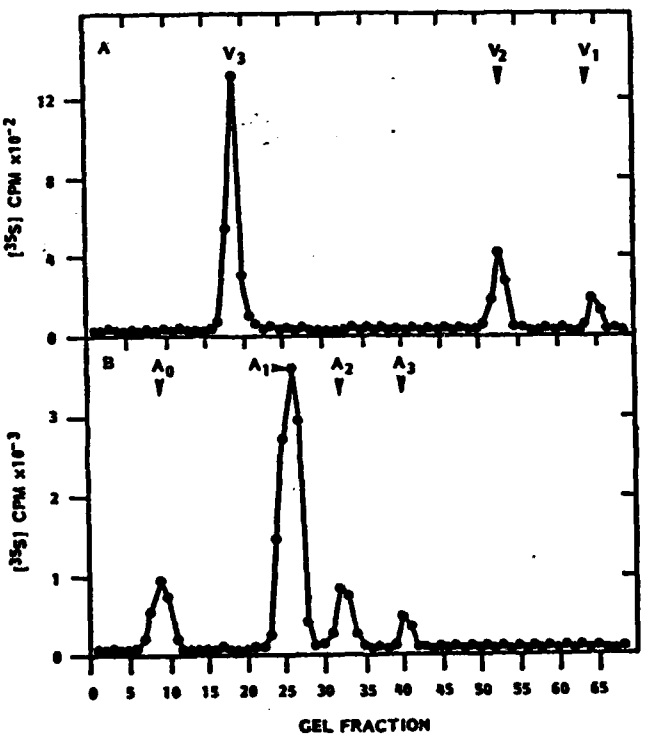
A
V_3
V_2
V_1
[^{35}S] CPM x10^{-2}
B
A_0
A_1
A_2
A_3
[^{35}S] CPM x10^{-3}
0 5 10 15 20 25 30 35 40 45 50 55 60 65
GEL FRACTION

Fig. 4

Table 4 Ability of antisera to precipitate intact Banzi virus

Labeled Components of mixture	% [^{35}S] cpm in aggregates formed after incubation with[c]	
	anti-Banzi viral serum	anti-mosquito cellular serum
Banzi virus$_{BHK}$[a]	55	<1
Banzi virus$_{A.albo}$[a]	59	45
A. albopictus hemagglutinin[b]	<1	53
A. albopictus hemagglutinin + unlabeled Banzi virus$_{BHK}$	<1	57
A. albopictus hemagglutinin + unlabeled Banzi virus$_{A.albo}$	<1	54
Banzi virus$_{BHK}$ + labeled A. albopictus hemagglutinin	56	<1

[a][^{35}S] methionine labeled Banzi virus grown in either BHK-21 cells or A. albopictus cells for 24 hours were purified by equilibrium density centrifugation. Virions present in regions of gradients corresponding to a density of 1.17 g/cc were pooled, diluted and centrifuged at 76,000 x g for 3.5 hours onto a pad of 100% glycerol. Virus was diluted so that 2 x 10^7 PFU were present in 0.5 ml of PBS.

[b]A. albopictus hemagglutinin obtained from uninfected mosquito cells which had been labeled with [^{35}S] methionine was purified and diluted to a final concentration of approximately 32,000 HA units per 0.5 ml of PBS.

[c]Virus and A. albopictus hemagglutinin were incubated with various antisera at 4°C for 30 minutes and immune aggregates which formed were collected and assayed for [^{35}S] radioactivity. Results are expressed as the percent of total radioactivity originally present in each assay.

Figure 5 Electrophoretic profiles of aggregates formed by the addition of various antisera to Banzi virus grown in A. albopictus cells. Labeled intact Banzi virus was incubated with antiviral serum (A) or with antisera to uninfected mosquito cells (B) as described in Table 4. The resulting aggregates were washed twice with PBS, resuspended in sodium phosphate buffer containing 8 M urea, 1% SDS and 1% mercaptoethanol and subjected to 8.5% polyacrylamide gel electrophoresis.

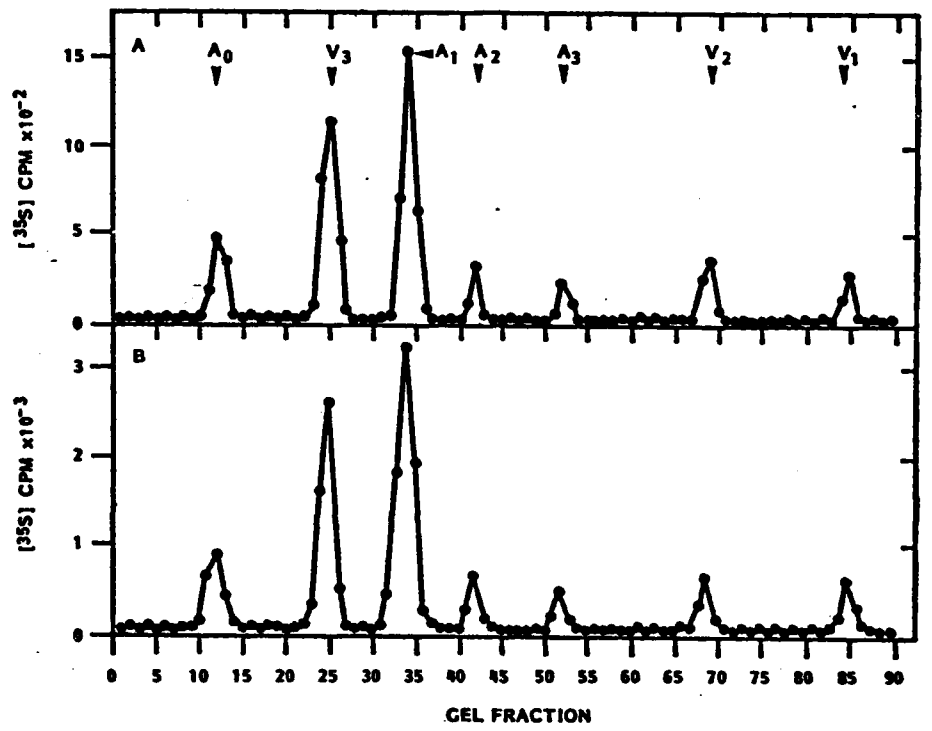

Fig. 5

Table 5 Adsorption of Banzi virus onto goose erythrocytes.[a]

Labeled Substance	pH of incubation	%[^{35}S] cpm associated with RBC
Banzi virus$_{BHK}$	6.5	72
	5.8	<1
Banzi virus$_{A.albo}$	6.5	81
	5.8	<1
A. albopictus hemagglutinin	6.5	<1
	5.8	83
Banzi virus$_{BHK}$ + unlabeled A. albopictus hemagglutinin	6.5	55
	5.8	<1
Banzi virus$_{A.albo}$ + unlabeled A. albopictus hemagglutinin	6.5	59
	5.8	<1
A. albopictus hemagglutinin + unlabeled Banzi virus$_{BHK}$	6.5	<1
	5.8	82
A. albopictus hemagglutinin + unlabeled Banzi virus$_{A. albo}$	6.5	<1
	5.8	87

[a][^{35}S] methionine labeled Banzi virus and A. albopictus cell hemagglutinin which had been purified by equilibrium density centrifugation as described in Table 4 were added to excess goose erythrocytes at the indicated pH. After 1 hour of incubation, erythrocytes were pelleted by centrifugation at 1000 x g for 15 minutes. The cell pellet was washed twice with PBS and then assayed for [^{35}S] radioactivity.

Table 6 Proteins associated with Banzi virus grown in mosquito cells for 1, 7 and 60 days[a]

Polypeptides	Relative amounts of proteins in Banzi virus grown in mosquito cells for following number of days		
	1	7	60
V_3	4.23	2.51	ND
V_2	1.0	1.0	1.0
V_1	0.80	0.81	0.77
A_0	0.92	0.95	0.9
A_1	5.54	7.01	8.96
A_2	0.92	1.01	0.95
A_3	1.0	0.8	1.0

[a]Banzi virus which had been grown in mosquito cells for 1, 7 or 60 days in the presence of [^{35}S] methionine were purified as described in the text. The amount of [^{35}S] methionine radioactivity in each peptide was determined after electrophoresis of disrupted virions on 8.5% polyacrylamide gels. Values were normalized to the amount of V_2 present in each virus preparation. ND, not detected.

Figure 6 Variation in pH optima for Banzi viral hemagglutination. Banzi virus were grown in A. albopictus cells for 24 hours (A), seven days (B) or for 12 days. Virions were purified as described in the legend to Figure 2. Virions which banded at a density of 1.17 g/cc were collected, dialyzed and precipitated by polyethylene glycol 6000. The resulting pellet was resuspended and used in HA assays performed at the indicated pH.

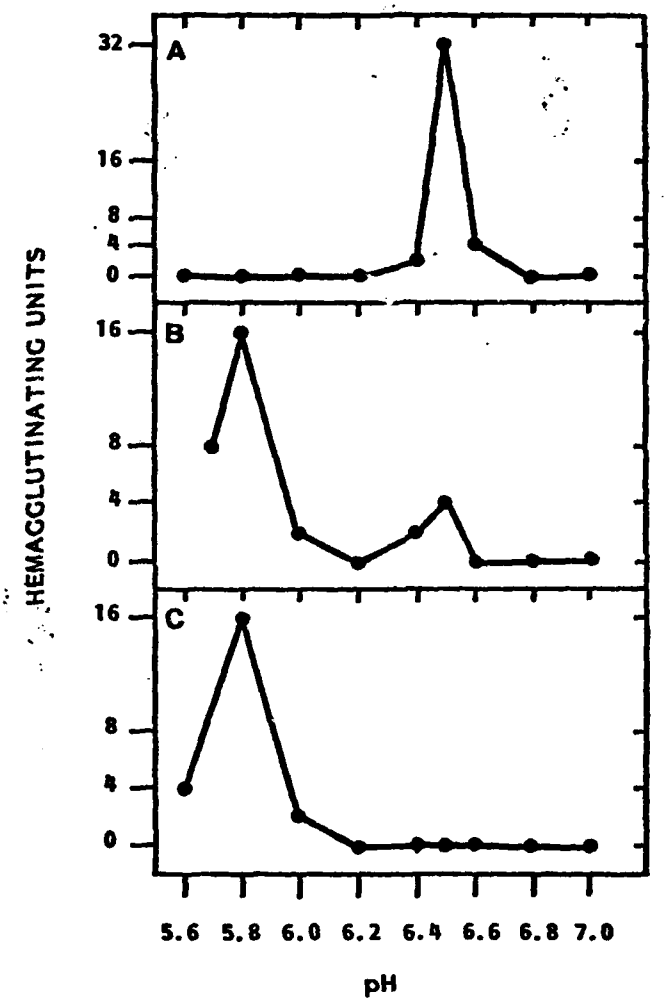
HEMAGGLUTINATING UNITS
32
16
8
4
0
A
16
8
4
0
B
16
8
4
0
C
5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0
pH

Fig. 6

Table 7 Effect of various antisera on the hemagglutinating activity of unpurified Banzi virus grown in various cells.

Serum	HAI titer of serum against virus grown in			
	BHK	A.al (1)	A.al (7)	A.al(7)→BHK
Anti-Banzi virus	512	8	4	1024
Anti-BHK cell	4	4	4	2
Anti-A. albopictus cell	4	1024	2048	4
Preimmune	4	4	4	4

[a]Banzi virions which was propagated in A. albopictus cells for 1 day and 7 days are designated as A.al (1) and A.al (7) respectively. Virus was grown in BHK cells for 24 hours.

[b]Anti-A. albopictus cell serum was prepared in rabbits as described in the text. Antiserum against BHK-21 cells was prepared in an identical manner whereas, anti-Banzi viral serum was prepared by immunization of rabbits with purified virus propagated in Vero cells. All sera were absorbed with fetal calf serum and complement was destroyed by heating at 56C for 30 minutes.

[c]HAI titer is calculated as the reciprocal of the highest dilution of serum which completely inhibited 8 HA unites of Banzi virus at pH 6.5.

Table 8 Effect of various antisera on hemagglutinating activity of purified Banzi virus propagated in various cell lines.

Serum	HAI titer against virus grown in				
	BHK	A.al (1)	A.al (7)		A.al(7)→BHK
			pH 6.5	pH 5.8	
Anti-Banzi virus	512	8	8	8	1024
Anti-BHK cell	4	4	4	4	4
Anti-A. albopictus cell	4	4096	8192	4096	4
Preimmune	4	4	4	4	4

1. Virus grown in A. albopictus cells and BHK cells were purified as described in legend to Figure 2. Virions (ρ=1.17 g/cc) were collected, pooled and used in HAI assays.

2. All HAI assays were conducted at pH 6.5 as described in Table 7 except when virus propagated in mosquito cells for 7 days were employed. In this instance HAI assays were conducted at pH 6.5 and pH 5.8 (final pH) since Banzi virus grown for 7 days in mosquito cells appear to have two pH optima (see Figure 6B).

Table 9 Effect of various treatments of anti-mosquito cell serum on hemagglutinating activity of Banzi virus grown in mosquito cells for 24 hours.

Treatment of serum	HAI Titer[d]
None	1024
Preadsorbed with mosquito cells[a]	4
Preadsorbed with BHK-21 cells[a]	1024
Preadsorbed with Banzi virus[a] infected BHK-21 cells	1024
Preincubated with purified[b] mosquito cell hemagglutinin	16
Preincubated with trypsinized[c] mosquito cell hemagglutinin	512
Preincubated with trypsin and soybean trypsin inhibitor	1024

[a]Two ml of anti-mosquito cell serum were mixed with one ml of 10^7 uninfected A. albopictus cells, of 10^7 uninfected BHK cells, or of 10^7 Banzi virus-infected BHK cells. After incubation at room temperature for 30 minutes the mixtures were subjected to centrifugation 800 x g for 15 minutes to remove cells. The procedure was reapeated once more. A sample of anti-mosquito cell serum received sufficient PBS to ensure that final volumes of all sera were equivalent.

When 2×10^7 Banzi virus-infected BHK cells was washed with two ml of PBS for 1.5 hours, the amount of virus released from these cells was insufficient to cause agglutination of goose erythrocytes.

[b]Two ml of anti-mosquito cell serum were mixed with 0.5 ml of purified A. albopictus hemagglutinin (500 ug of protein). After incubation at room temperature for 30 minutes, the mixture was stored for 16 hours at 4°C. Immune precipities which had formed were removed by centrifugation. This procedure was repeated twice. Previous experiments had shown that anti-mosquito cell serum precipitates the proteins comprising the A. albopictus hemagglutinin. As a control, 0.5 ml of PBS was used instead of the purified A. albopictus hemagglutinin. The HAI of this control was 1024 and is not included in Table 9.

[c]Mosquito cell hemagglutinin was treated with trypsin (1 mg) for 2 hours at 37°C prior to use. Reaction was terminated by the addition of soybean trypsin inhibitor.

[d]All assays were performed at pH 6.5.

Table 10 Survival of mice injected with unpurified Banzi virus pretreated with anti-mosquito cell serum

Virus[a]	Antiserum[b]	Deaths (#/total)[c] Expt 1	Expt 2
A. al (1)	-	20/20	20/20
	+	4/20	19/20
A. al (7)	-	12/12	20/20
	+	0/12	2/20
A. al (60)	-	20/20	20/20
	+	0/20	0/20
BHK	-	10/10	
	+	10/10	

[a]Banzi virus propagated in mosquito cells for 1, 7 and 60 days were used. Virus was grown in BHK cells for 24 hours.

[b]An amount of virus equivalent to approximately 10 LD_{50} was mixed with PBS or anti-mosquito cell serum at room temperature for 30 minutes prior to injection intraperitoneally into groups of random-bred mice (10-14 days of age). The inocula (virus + PBS) was back titrated at the same time to accurately determine the amount of virus; results shown that 9.8 LD_{50} of virus was injected.

[c]Mice were examined on a daily basis for 21 days after injection.

Table 11 Survival of mice injected with purified Banzi virus mixed with anti-mosquito cell serum.[a]

Virus	Antisera	Deaths no. of mice/total
A. al (1)	-	11/20
	+	0/20
	-	20/20
	+	4/20
A. al (7)	-	9/18
	+	0/20
	-	12/12
	+	0/12
BHK	-	10/10
	+	10/10

[a]The source of virus is described in Table 8. Each virus preparation was purified by polythylene glycol precipitation and equilibrium density gradient centrifugation for 40 hours.

Table 12 Ability of various antisera to neutralize the infectivity of unpurified Banzi virus.[a]

	Neutralization titer of serum against virus grown in			
	BHK	A. al(1)	A. al(7)	A. al(60)
Anti-Banzi virus	1024	512	256	128
Anti-mosquito cell	4	16(4)	256	512
Anti-BHK cell	4	4	2	4
Preimmune	4	2	4	4

[a]Preparation of virus is described in Table 10. Two different preparations of Banzi virus grown in mosquito cells for 24 hours were used; significant differences in results between the two preparations are noted in parenthesis.

Neutralization tests were performed by mixing 200 PFU of Banzi virus in 0.2 ml of lactalbumin hydrolysate with 0.2 ml of two-fold serial dilution of various sera. After incubation at room temperature for 30 minutes, 0.2 ml of the mixture was plated on monolayers of BHK-21 cells. Three days after incubation at 37°C, the monolayers were stained with neutral red and plaques were scored. Anti-Banzi viral serum was initially diluted 1:5 before use.

Table 13 Ability of anti-mosquito cell serum to neutralize infectivity of purified Banzi virus.

Serum	Neutralization titer of sera against virus grown in			
	BHK	A.al(1)	A.al(7)	A.al(7) →BHK(2)
Anti Banzi virus	1024	512	512	1024
Anti-mosquito	4	16(8)	256	4
Anti-BHK cell	4	4	2	4
Preimmune	4	2	4	4

[a]Source of virus is described in Table 8. Experimental protocol was identical to that described in Table 12.

Table 14 Effect of various treatments of anti-mosquito cellular serum of Banzi viral infectivity[a]

Treatment of serum	Neutralization titer of serum against virus grown in			
	BHK	A.al(1)	A.al(7)	A.al(60)
None	4	8	256	256
Preincubated with mosquito cells	4	4	8	8
Preincubated with mosquito cell hemagglutinin	4	4	32	32
Preincubated with trypsinized mosquito cell hemagglutinin	4	4	256	128

[a]Treatment of anti-mosquito cell serum is described in Table 9. Neutralization assays conducted as described in Table 12.

Table 15 Mosquito cell antibody titers in serum of mice immunized with A. albopictus cells.

Route of Immunization[1]	No. of injections[2]	HAI titer[3]
I.P.	0	0
	1	0
	3	64
	5	1024
I.V.	0	0
	1	2
	3	32
	5	1024

1. Mice were immunized with 1 ml of 10^7 washed, disrupted A. albopictus cells either intraperitoneally (i.p.) or intravenously (i.v.)

2. Injections were given at weekly intervals. Mice were sacrificed 7 days after the last injection and serum was collected.

3. The assay to determine relevant antibody titers was to determine the ability of serum to inhibit hemagglutinating activity of Banzi virus grown in A. albopictus cells for 24 hours. The HAI titer is defined in Table 7.

Table 16 Resistance to challenge with Banzi virus induced by prior immunization of mice with A. albopictus cells

Virus dose (LD_{50})	Survival after 21 days No. of mice/total	
	Unimmunized	Immunized
1	3/10	22/22
10	0/10	24/25
100	0/10	21/24

1. Groups of mice (10-14 days old) were immunized with 10^7 disrupted A. albopictus cells i.p. once a week for five weeks. Unimmunized mice were injected with PBS. Thirty-five days after the initial injection, immunized and unimmunized mice were injected i.p. with various doses of Banzi virus grown seven days in A. albopictus cells. Virus innocula was back titrated at the time of the experiment.

Table 17 Ability of various immunogens to protect mice from challenge with Banzi virus[1]

Immunogen	Source of virus used as challenge	Survival of mice 21 days after challenge No. of mice/total Expt 1	Expt 2
None	A.al	0/5	1/20
	BHK	0/5	0/20
A. albopictus cells	A.al	15/16	28/30
	BHK	0/16	0/20
M and M medium	A.al	0/9	1/25
	BHK	0/7	0/25
BHK-21 cells	A.al	0/15	0/10
	BHK	0/13	1/10
A. albopictus hemagglutinin	A.al	19/20	30/30
	BHK	0/20	2/30

1. Mice (10-14 days old) were immunized i.p. with 10^7 disrupted A. albopictus cells or BHK-21 cells, with 0.5 ml of PBS (control) or of M and M medium or with 100 ug of A. albopictus hemagglutinin which was purified as described in the text. Injections were given on day 0, 7, 21. On day 27, 100 LD_{50} of virus which was grown seven days in mosquito cells or in BHK-21 cells (BHK) were injected i.p. Virus innocula was back titrated at time of experiment.

Table 18 Survival of mice immunized with mosquito cells or with BHK cells after challenge with arboviruses[a]

Challenge virus	Cell line for viral growth	Cell line used as immunogen	No. of survivors/total (%) Expt 1	Expt 2
Eastern equine encephalitis virus	A. albopictus	A. albopictus	16/20 (80)	15/19 (79)
		BHK-21	0/10 (0)	0/5 (0)
	BHK-21	A. albopictus	0/14 (0)	0/20 (0)
		BHK-21	0/12 (0)	0/15 (0)
Germiston virus	A. albopictus	A. albopictus	0/20 (0)	0/10 (0)
		BHK-21	0/10 (0)	0/15 (0)
	BHK-21	A. albopictus	0/10 (0)	0/10 (0)
		BHK-21	0/10 (0)	0/10 (0)
Japanese encephalitis virus	A. albopictus	A. albopictus	26/30 (87)	13/15 (87)
		BHK-21	1/20 (5)	0/15 (0)
	BHK-21	A. albopictus	0/15 (0)	1/15 (6)
		BHK-21	0/15 (0)	0/15 (0)
Vesicular stomatitis virus	A. albopictus	A. albopictus	2/30 (7)	
		BHK-21	1/20 (5)	
	BHK-21	A. albopictus	0/20 (0)	
		BHK-21	1/15 (7)	

[a]The various viruses were grown for 24 hours in BHK-21 cells or for 5 days in A. albopictus cells. Immunization of mice with cell lines is described in Table 17. Mice were challenged with 10 LD_{50} (back titrated at the time of experiment) and held for 21 days after viral challenge.

Table 19 Survival of mice immunized with monoclonal A. albopictus cells after injection with Banzi virus[a]

Cell line for viral growth	Immunogen	No. of survivors/total %
BHK-21	BHK-21 cells	0/10 (0)
	Dulbecco's medium	1/10 (10)
	A. albopictus cells	1/10 (10)
A. albopictus	BHK-21 cells	0/20 (0)
	Dulbecco's medium	1/10 (10)
	A. albopictus cells	29/30 (97)

[a]The experimental protocol is as described in Table 17, except that the monoclonal A. albopictus cell line was used. Mice were challenged with 10 LD_{50} of virus grown in mosquito cells for 7 days or in BHK-21 cells for 24 hours. The number of surviving mice after 21 days post-challenge is given.

Figure 7 Growth of Banzi virus in A. albopictus cells. Mosquito cells were infected with Banzi virus at a MOI of 50. At the times indicated the titer of virus present in the medium was determined by plaque assays on BHK-21 cells, (A). In addition, the number of infected cells was determined by infectious center assays employing BHK-21 cell as the host cell, (B). Infected cultures were passaged every seven days.

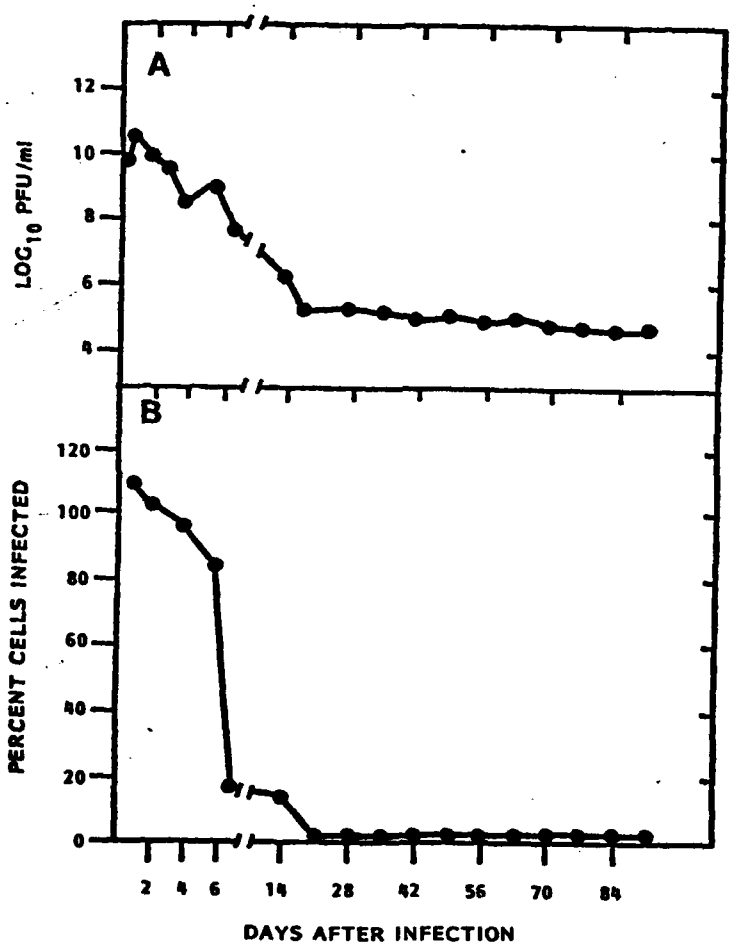
A
B
12
10
8
6
4
LOG_{10} PFU/ml
120
100
80
60
40
20
0
PERCENT CELLS INFECTED
2
4
6
14
28
42
56
70
84
DAYS AFTER INFECTION

Fig. 7

Table 20 Growth of togaviruses in A. albopictus cells infected with Banzi virus

Virus	Cell line	Yield after 24 hr (PFU/ml)
None	Fresh	0
	P.I.	5×10^4
Banzi virus	Fresh	1.2×10^9
	P.I.	6.1×10^4
Japanese encephalitis virus	Fresh	6.8×10^7
	P.I.	5.9×10^7
Eastern equine encephalitis virus	Fresh	2.5×10^{10}
	P.I.	1.8×10^{10}

Uninfected (Fresh) mosquito cells or mosquito cells (PI) infected with Banzi virus for 60 days were infected with the indicated virus (MOI=50). The yield of virus after 24 hours of infection was determined by plaque assays on BHK cells at 37°C.

Table 21 Presence of Banzi viral antigens in infected mosquito cells as determined by immunofluorescence assays

Serum	Cells(% fluorescing)		
	uninfected	Acutely infected	Persistently infected
Anti-Banzi virus	0	85	91
Anti-Banzi virus preadsorbed with infected mosquito cells	0	0	0
Normal	0	0	0

[a]Uninfected mosquito cells or mosquito cells infected for 24 hours or 60 days with Banzi virus were fixed with acetic acid; ethanol and then reacted with anti-Banzi viral serum. After washing, the cells were treated with fluorescinated goat-anti-rabbit IgG at room temperature. The percent of cells fluorescing were determined. A minimum of 300 cells were examined.

Figure 8 Presence of viral antigens in mosquito cells infected with Banzi virus. Mosquito cells were infected with Banzi virus and prepared for indirect immunofluorescence as described in Tabel 21. (A) Cells infected with virus for 24 hours. (B), Cells infected for 60 days.

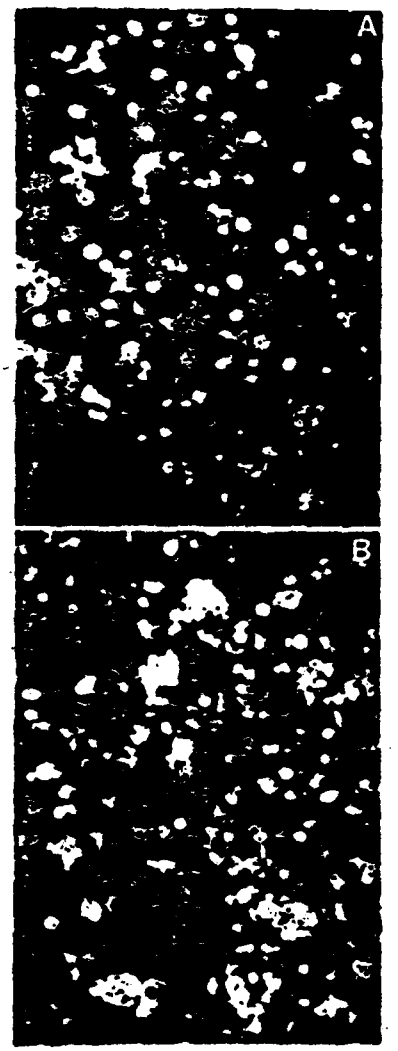

Fig. 8

Figure 9 Attachment of soluble viral proteins to uninfected mosquito cells. Filtrate of medium of mosquito cells persistently infected with Banzi virus (60 days) was incubated with uninfected mosquito cells. After 2 hours of incubation at 28C, cells were examined by immunofluorescence procedures as described in Table 21. Cells treated with filtrates of medium of uninfected mosquito cells did not react with anti-Banzi viral serum (data not shown). (A), uninfected cells treated with filtrate (B), Mosquito cells infected with Banzi virus for 60 days.

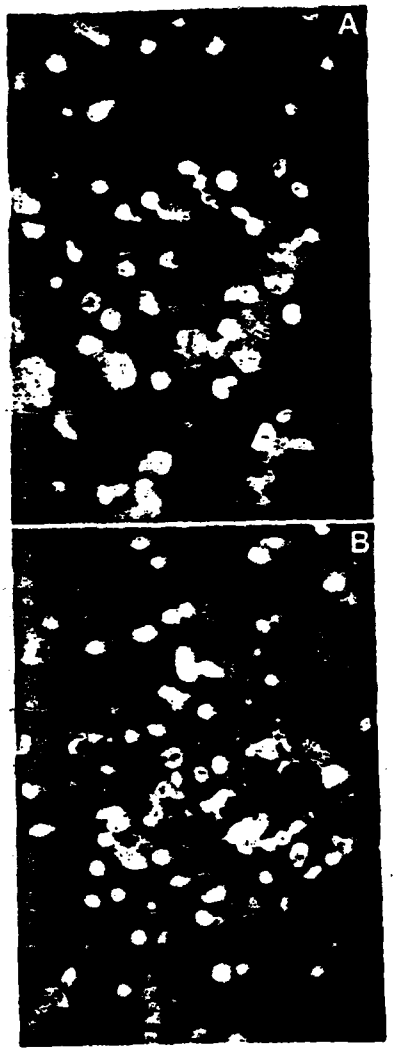

Fig. 9

Table 22 Effect of filtrates of culture medium of infected mosquito cells on the replication of Banzi virus

Treatment of mosquito cells	PFU/ml	%
Filtrate of M&M medium	8.1×10^8	100
Filtrate of medium of uninfected mosquito cells	8.0×10^8	99
Filtrate of medium of acutely infected cells	6.6×10^8	81
Filtrate of medium of persistently infected cells	1.6×10^4	0.002
Filtrate of medium of persistently infected cells pretreated with anti-Banzi viral serum	7.5×10^8	93
Filtrate of medium of persistently infected cells pretreated with anti-mosquito cell serum	3.2×10^4	0.004

1. Mosquito cell growth medium and medium of uninfected cells and medium of cells infected with Banzi virus for 24 hours (acutely infected) or for 60 days (persistently infected) were subjected to filtration as described in the text. After sterilization, aliquots of filtrates were placed on monolayers of uninfected cells and after 4 hours of incubation at 28°C each monolayer was infected with Banzi virus (MOI=20). After 24 hours of infection, the amount of extracellular virus was determined by plaque assays.

2. Medium of persistently infected mosquito cells were treated with antiserum against either Banzi virus or mosquito cells. After incubation for 2 hours at 4°C, goat-anti-rabbit serum was added and incubation was contained for 2 hours. The treated medium was then filtered and use. No evidence of residual antibodies to virus or mosquito cells in resulting filtrates could be obtained.

Figure 10 Effect of soluble viral proteins on attachment of Banzi virus to A. albopictus cells. Monolayers of A. albopictus cells were treated with filtrates of media of uninfected mosquito cells or of mosquito cells infected with Banzi virus for 60 days. After two hours of incubation at 28C, cell cultures were given equal amounts of [^{32}P]-labeled Banzi virus (30,000 cpm). At the times indicated, monolayers were washed and the amount of cell-associated radioactivity was determined. Cells treated with medium of uninfected cells (●); cells treated with medium of persistently infected cells (○).

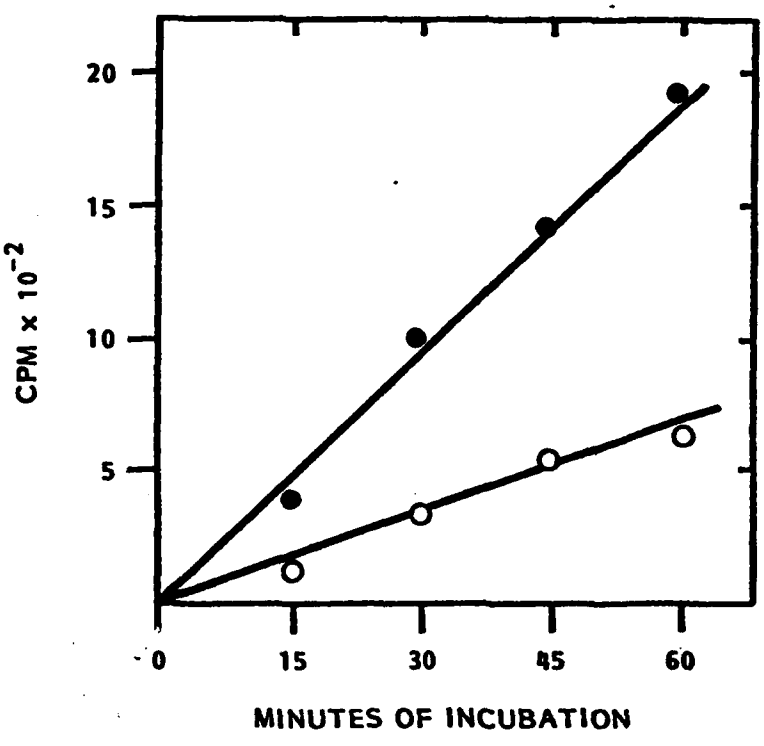

Fig. 10

Table 23 Comparison of the ability of various agents in medium of persistently infected mosquito cells to inhibit Banzi viral replication.

Treatment of mosquito cells	PFU/ml	%
Filtrate of M&M medium	6×10^8	100
Filtrate of medium of uninfected cells	6.1×10^8	102
Filtrate of medium of PI cells	3.0×10^4	0.005
Dialysate of M&M medium	7.5×10^8	100
Dialysate of medium of uninfected cells	7.4×10^8	99
Dialysate of medium of PI cells	4.5×10^4	.006
Dialyzed filtrates of M&M medium	1.2×10^9	100
Dialyzed filtrates of medium of uninfected cells	1.1×10^9	92
Dialyzed filtrates of medium of PI cells	6.2×10^8	52

Filtrates were obtained as described in the text. Aliquot of filtrate (25 ml) was dialyzed against 50 ml of M and M medium for 18 hours. The resulting dialysate was lyophilized and reconstituted in 12.5 ml of M and M medium. The filtrates (1 ml) were then exhaustively dialyzed against 20 liters M and M medium. The original filtrate, dialysate and dialyzed filtrate were sterilized by Millipore filtration, and used to determined their ability to inhibit Banzi viral growth in mosquito cells as described in Table 22. PI; persistently infected.

Table 24 Effect of various antisera on activity of antiviral agent

Treatment of cells	Yield (PFU/ml)	%
Dialysate of M&M medium	6.6×10^8	100
Dialysate of medium of PI cells	5.5×10^6	0.8
Dialysate of medium of PI cells pretreated with anti-mosquito cell serum	5.0×10^6	0.75
Dialysate of medium of PI cells pretreated with anti-Banzi viral serum	6.4×10^8	97

Mosquito cell medium or medium of Banzi virus persistently infected mosquito cells were placed in a dialysis sack and dialysed against 2 volumes of M and M medium. In addition medium of persistently infected (PI) cells was incubated with anti-mosquito cell serum or anti-Banzi viral serum for 2 hours at 37°C prior to transfer to dialysis sacks. After dialysis for 18 hours, the dialysates were sterilized and used to determine their effects on Banzi viral replication as described in Table 22.

Figure 11 Effect of dilution of the activity of the antiviral agent. Media of uninfected mosquito cells and of mosquito cells infected with Banzi virus for 60 days were dialyzed against two volumes of M and M medium. The resulting dialysates were sterilized by filtration and diluted with M and M medium. Uninfected mosquito cells were treated with various dilutions of the dialysates for 4 hours prior to infection with Banzi virus (MOI=100). After 24 hours of infection, the amount of extracellular virus produced in cells pretreated with dialysates of medium of persistently infected mosquito cells was determined and compared to virus produced in cells treated with dialysates of medium of uninfected cells.

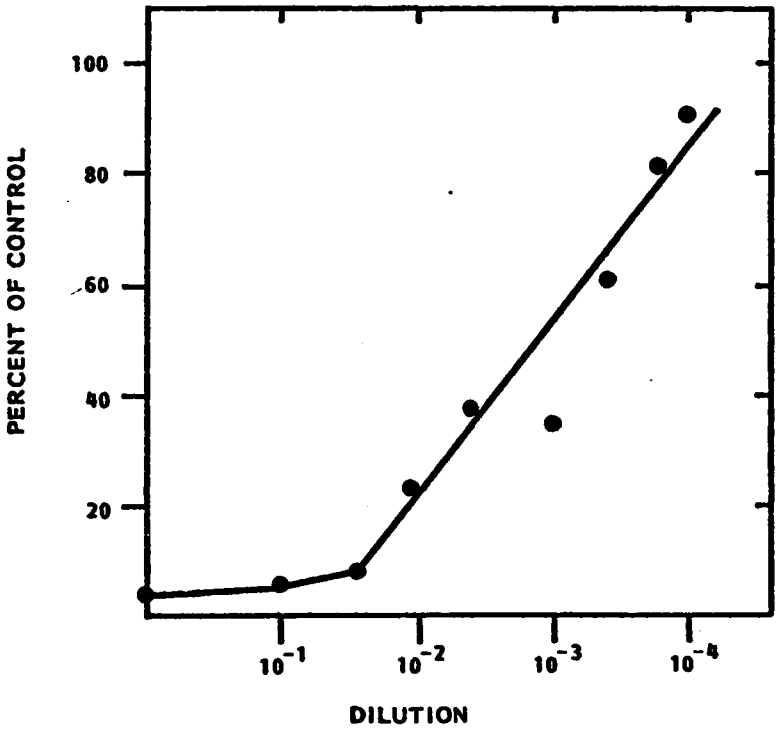

Fig. 11

Table 25 Effect of antiviral factor on number of infectious centers

Treatment	Infectious centers % Expt 1	Expt II
Dialysate of medium of uninfected A. al cells	85	94
Dialysate of medium of PI cells	4	5
Dialysate of medium of PI cells pretreated with anti-Banzi viral serum	83	87

Dialysates were prepared as described in Table 24 and added to monolayers of uninfected mosquito cells for 4 hours. Treated mosquito cells were infected with Banzi virus for (MOI=100) and incubated at 28°C for 24 hours. The number of cells capable of releasing infectious virus was determined by infectious center assays.

Table 26 Effect of antiviral agent on replication of togaviruses in mosquito cells

Virus	Yield of virus (PFU/ml) after 24 hours of infection in cells treated with dialysates of	
	Uninfected A.al cells	P.I. A.al cells
Banzi virus	1.2×10^9	7.2×10^6
Japanese encephalitis virus	6.9×10^8	6.5×10^8
Eastern equine encephalitis virus	8.1×10^8	8.5×10^8

Uninfected mosquito cells were treated for 4 hours with dialysates of medium of either uninfected mosquito cells or mosquito cells infected with Banzi virus for 60 days. Cells were infected with the indicated virus at a MOI of 50 and incubated for 24 hours at 28°C. The amount of virus in the medium was determined by plaque assays.

Figure 12 Attachment of soluble viral proteins of molecular weight of 12,000 daltons or less to uninfected mosquito cells. Experimental protocol was identical to that described in Figure except that dialysates of medium of persistently infected mosquito cells were used. Cells treated with dialysates of uninfected mosquito cells did not react with anti-Banzi viral serum (data not shown).

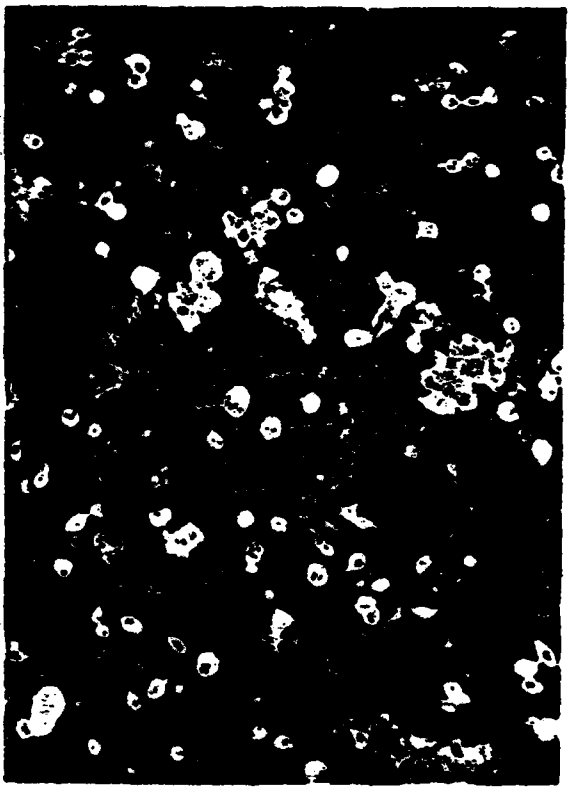

Fig. 12

Figure 13 Time course of appearance of antiviral activity. Mosquito cells were infected with Banzi virus at a MOI of 50. At the indicated times medium was collected and assayed for the presence of antiviral activity as described in the legend to Figure 12. Experiment was done in duplicate.

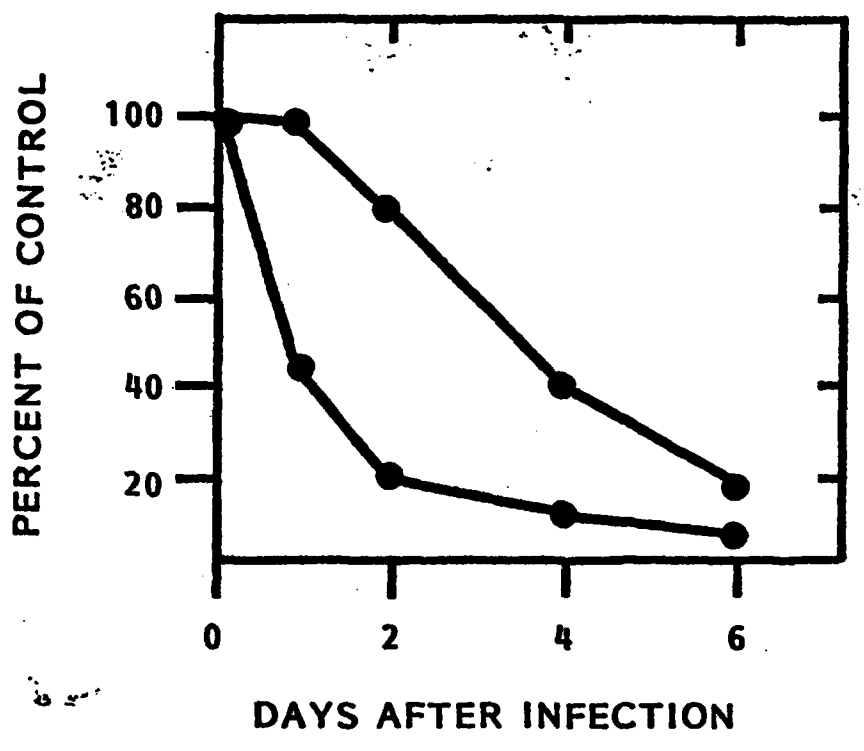

Fig. 13

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